

Isolation Distances for Transgenic Alfalfa Seed Production in the Pacific Northwest

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ABSTRACT

Alfalfa (*Medicago sativa* L.) was the first perennial crop genetically engineered (GE) to resist glyphosate herbicide. Since alfalfa is insect pollinated, the potential for transgene flow and occurrence of adventitious presence (AP) in conventional varieties is a concern, especially for organic and export producers. In response, the industry developed seed production isolation distances. However, no landscape-scale studies have been conducted to validate industry standards. Our objective was to quantify the extent of gene movement from GE seed fields to commercial conventional seed fields on a landscape level to provide the industry with information to support coexistence strategies. Alfalfa seed fields were mapped in the Touchet area, Walla Walla Valley, Washington, during spring 2013. Fourteen conventional seed fields located at various distances from GE sources were identified, harvested, and tested for the presence of the GE trait. Although other variables affect gene flow, this article focuses on geographic distance, since this variable can be easily controlled by growers and industry. Using distance as the predictor variable, we modeled the relationship between AP proportion and distance to GE source fields at 0.1, 0.5, and 0.9% AP threshold levels using quadratic regression. As distance increased, transgene flow generally decreased. At a threshold level of 0.9% AP, an isolation distance of 330 m between conventional and GE fields is needed to ensure that at least 95% of the samples fall below this threshold level. Spatial prediction maps were consistent with our statistical analysis. At an average distance of 2.2 km, AP was 0.05%, and this decreased to 0.03% at an average distance of 8.8 km from GE seed fields.

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Abbreviations: AB, alkali bees; ALCB, alfalfa leafcutter bees; AOSCA, Association of Seed Certifying Agencies; AP, adventitious presence; ASSP, Alfalfa Seed Stewardship Program; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GE, genetically engineered; GOZ, Grower Opportunity Zones; HB, honey bees; NAFA, National Alfalfa and Forage Alliance; PCR, polymerase chain reaction.

POLLEN-MEDIATED GENE FLOW occurs naturally within and between populations, and occasionally even between species (Levin and Kerster, 1974), and is strongly influenced by distance (Papa and Gepts, 2003; Beckie and Hall, 2008; Heuberger et al., 2010). Seed producers routinely use standard isolation distances to maintain varietal purity by minimizing pollen-mediated gene flow between different varieties of the same crop (Beckie and Hall, 2008). Industries with genetically engineered (GE) cultivars are challenged to strengthen these strategies to support the coexistence of GE and non-GE supply chains, since adventitious presence (AP) of GE traits in conventional seed has negative economic consequences (Greene et al., 2016; NASEM, 2016). Research efforts have focused on understanding the pollen-mediated gene flow of GE traits in maize (*Zea mays* L.; Weber et al., 2007), canola (*Brassica napus* L.; Beckie et al., 2003; Beckie

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and Hall, 2008), plum (*Prunus domestica* L.; Scorza et al., 2013), cotton (*Gossypium hirsutum* L.; Heuberger et al., 2010), alfalfa (*Medicago sativa* L.; Greene et al., 2015), and sugar beets (*Beta vulgaris* L.; Darmency et al., 2009) to help develop coexistence practices that allow growers to produce either GE or non-GE varieties without negatively affecting neighboring growers.

Alfalfa is the fourth most valuable field crop in the United States, following corn, soybean [*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.) (USDA-NASS, 2017). Between 2005 and 2007, the USDA Animal and Plant Health Inspection Service (USDA-APHIS, 2005) deregulated GE glyphosate-resistant alfalfa, making it the first major perennial GE crop available for commercial cultivation. It was deregulated a second time in 2011. This was followed by the deregulation of GE low-lignin alfalfa in 2014 (USDA-APHIS, 2014). The United States is the world's leading exporter of alfalfa seed; nearly 33% of the crop is exported (Van Deynze et al., 2008). According to Putnam et al. (2016), 4.7% of the alfalfa hay produced in the United States is exported. For seven western states (Arizona, California, Idaho, Nevada, Oregon, Utah, and Washington), the percentage rises to 15.3%. The rate is even higher for some regions, such as the Columbia Basin in Washington–Oregon and the southern valleys of California. Although the vast majority of hay is sold domestically, export markets can provide profitable returns for alfalfa growers. Organically produced hay is also important. In 2011, ~171,182 ha of organic alfalfa hay and silage were produced (USDA-ERS, 2011), which is ~2.1% of total US production.

At present the majority of US export markets are AP sensitive (i.e., they do not want GE products). Consequently, growers in areas that export hay have been proactive in developing coexistence practices (Cornish et al., 2014; McCaslin and Van Deynze, 2014; Putnam et al., 2014). Understanding alfalfa pollen-mediated gene flow is complicated since alfalfa, while mainly an insect cross-pollinated crop, can also be self-pollinated. Commercially produced alfalfa seed is pollinated primarily by managed pollinators, typically honey bees (HB, *Apis mellifera* L.), alfalfa leafcutter bees (ALCB, *Megachile rotundata* Fabricius), and alkali bees (AB, *Nomia melanderi* Cockerell). Wild bees such as bumble bees (*Bombus* spp.) also pollinate alfalfa (Rincker et al., 1988; Brunet and Stewart, 2010).

Long-standing isolation standards are in place for conventional alfalfa seed production. Depending on field size (<2.02 or >2.02 ha), the isolation distance for foundation seedlots is 274 and 182 m, respectively; for registered seedlots, it is 137 and 91 m, respectively, and for certified seedlots, it is 50 m, regardless of field size (https://www.aphis.usda.gov/biotechnology/downloads/alfalfa/gealfalfa_deis.pdf). The National Alfalfa and Forage Alliance (NAFA) best practices for GE seed production take into

account pollinator foraging distances for different bee species and specify that GE seed production fields be separated from conventional seed fields by 274 m for pollination by ALCB, by 1.6 km for pollination by AB, and by 4.8 km for pollination by HB, to limit transgene flow to conventional seed fields (Cornish et al., 2015b). The Association of Official Seed Certifying Agencies (AOSCA) Alfalfa Seed Stewardship Program (ASSP), which provides an Identity Preserved certificate for AP sensitive seed lots, specifies ASSP fields be separated from GE alfalfa fields by 8 km.

Several research-scale studies provide the scientific basis for the coexistence strategies developed and implemented by NAFA and AOSCA (Cornish et al., 2014; McCaslin and Van Deynze, 2014; Putnam et al., 2014; AOSCA, 2015). Fitzpatrick et al. (2003) reported that with ALCB, transgene flow was 0.5% at 305 m, <0.2% at 457 m, and undetectable at 610 m. Teuber et al. (2004) and Van Deynze et al. (2004) reported that with HB, transgene flow decreased with distance, but they could still detect low levels (<0.03%) at 4 km. Teuber et al. (2007) reported that when both HB and ALCB were used as pollinators, transgene flow was <0.2% at 1.6 km, <0.03% at 4.8 km, and nondetectable at 8.1 km. These studies were conducted mainly in California, with a few in Idaho. The AP threshold levels in conventionally produced alfalfa seed vary domestically by seed class (certified, registered, and foundation) and internationally by country. Although there is no uniform AP threshold for all AP-sensitive seed markets, many AP-sensitive markets have adopted a “nondetect” standard, a standard that certifies there is no detection of the GE trait in a seed sample of a certain size. According to NAFA, seed containing <0.1% AP meets the nondetect standard.

Since 2011, NAFA has facilitated the establishment of Grower Opportunity Zones (GOZ), areas where the production of GE or AP sensitive alfalfa seed is concentrated (https://www.alfalfa.org/bio_growerzones.php; Cornish et al., 2015c). Genetically engineered GOZs allow the production of GE and conventional AP-tolerant seed lots. Adventitious presence-sensitive GOZs support the production of AP-sensitive seed lots, where GE seed production is not allowed although GE hay production is permitted (Cornish et al., 2015c). An online field pinning map has been set up to help seed producers identify locations that support the purity of GE and non-GE seed (<https://ccia.ucdavis.edu/pinning-maps>). More recently, NAFA has developed a set of recommendations for AP-sensitive seed producers (Cornish et al., 2015a) and best management practices for GE seed producers (Cornish et al., 2015a). The industry has also concluded that AP levels of <0.1% using standard industry tests meet the nondetect standard.

Although the industry is monitoring AP using a third-party process, there has been no landscape-level study to

examine transgene flow following the second period of deregulation in 2011. Studies of AP in corn and canola (*Brassica campestris* L.) have emphasized the importance of sampling at commercial-field and landscape scales to understand how transgene flow occurs across the environment (Rieger et al., 2002; Allnutt et al., 2008). Amand et al. (2000) found significant differences in pollen-mediated gene flow when they compared research-scale and commercial-scale plots of alfalfa. They found evidence of their marker trait 200 m away from research-scale plots and 1 km from commercial-scale plots. Alfalfa studies used to develop coexistence strategies have been largely limited to research-scale experiments (Fitzpatrick et al., 2003, 2007a, 2007b; Teuber et al., 2004, 2007). Research has also focused on alfalfa production using ALCB and HB as pollinators in California.

Here, we evaluated pollen-mediated gene flow of the CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) transgene into commercial alfalfa seed production fields in an area designated as a GE-GOZ that encompasses 270 km². We developed spatially explicit statistical models to characterize transgene flow at the landscape level. Our main objective was to predict the distance between GE and conventional alfalfa seed fields that lead to AP levels of less than 0.1, 0.5, and 0.9% in conventional fields.

MATERIALS AND METHODS

Study Fields and Sampling Design

The Walla Walla Valley and Columbia Basin in Washington State are important areas for alfalfa seed production. Our study fields were located in the Touchet-Gardena area of the Walla Walla Valley in Washington State and Oregon. The Touchet area has been designated a GE GOZ where both GE and conventional alfalfa seed is produced (<http://alfalfa.org/pdf/Walla%20Walla%20map%20with%20physical%20description.pdf>). Genetically engineered and conventional seed fields were mapped in the Touchet area (center of the study area: 46.023° N, 118.6154° W) west of the town of Walla Walla Valley, WA, using ArcGIS 10.2 (ESRI, 2013). The valley is characterized by average seasonal temperatures ranging from 5.1 to 18.0°C, average relative humidity of 65%, total precipitation of 143 mm, average wind speed of 2.5 m s⁻¹, maximum wind gusts of 26.5 m s⁻¹, and average wind direction coming from the southwest (<https://weather.wsu.edu/>).

We selected 14 commercial conventional seed fields located at different distances from GE seed fields (Fig. 1). Since one of the fields (Field 1) was planted with three alfalfa varieties, we had a total of 16 study fields. The most distant conventional field was 11 km away from the nearest GE field. Conventional and GE fields were planted at the same time and flowering overlapped. Alfalfa leafcutter bees and AB are the primary pollinators managed by growers in Walla Walla Valley. Growers place ALCB in the field from May to July. Normal peak activity of AB in Touchet Valley is from mid-June to mid-July (Johansen et al., 1982). Alkali bees are ground-nesting bees, and Fig. 1 shows the location of AB beds in the study area. Honey bees are

not used in the Touchet area, and county ordinance limits the number of HB hives in the area. Honey bees learn to abscond nectar from alfalfa without tripping the blossom; hence, seed growers in Walla Walla County consider HB a nuisance pest.

Transgene flow from GE fields to conventional fields was monitored in 2013. Sampling protocols for the detection and quantification of GE contamination are well established (Redmund et al., 2001; Whitaker et al., 2001; Laffont et al., 2005; Emslie et al., 2007; Hernández-Suárez et al., 2008). Although the procedures to detect high levels of GE contamination reliably are well understood, the quantification of low levels of contamination is much more challenging from a resource standpoint, since more seeds need to be tested. In fields located ~250 m or more from a GE source, where we would expect low levels of AP, we focused on sampling field edges where gene flow rates have been found to be relatively high compared with field centers (Llewellyn et al., 2007; Heuberger et al., 2010). Most samples were obtained directly from combines when growers harvested in August, September, and October. Eleven of the study fields were sampled along the one or two edges nearest to a GE seed field, and samples were taken every 30 m. Five study fields located <250 m from a GE source were intensively sampled. In addition to sampling all four edges, seed samples were obtained every 15 m from transects that went across the field (Fig. 1). Hand samples were obtained along the edge of two fields.

Testing for Transgene Presence

Harvested seed samples were tested for the presence of the EPSPS protein. Seed samples were cleaned, scarified, and assessed for AP using a seedling germination assay (Boyle et al., 2016). Using preliminary data from three fields, we estimated the seed sample size needed to quantify AP to a 0.1% level with confidence intervals of 90, 95, and 99%. Based on our preliminary analysis, we tested 7200 seeds per sample, which allowed us to measure AP to 0.042%. Resistant seedlings identified from the seedling germination assay were tested to confirm the presence of the transgene using AgraStrip RUR TraitChek test strips (Romer Labs), which provide a qualitative threshold test based on CP4 EPSPS-specific antibodies coupled to a color reagent. Each seedling was ground with 0.5 mL of distilled water in a 1.5-mL Eppendorf tube. The slurry was stirred using a disposable stirrer, and the TraitChek test strip was placed in the tube. After 5 min, samples were scored as either positive or negative for the transgene based on the presence or absence of a colored test line (Greene et al., 2015; Boyle et al., 2016).

Real-time polymerase chain reaction (PCR) was also used to confirm the presence of the transgene (Watrud et al., 2004; Greene et al., 2015). Positive seedlings identified from the seedling germination assay were placed in 96-well plates and numbered accordingly. The seedlings were freeze dried, and genomic DNA was extracted from the lyophilized samples. Genomic DNA was initially purified from pulverized freeze-dried seedlings using the Rapid One-Step Extraction (ROSE) method (Steiner et al., 1995). The lyophilized seedling samples were placed in 2-mL grinding tubes (Daigger BIO4050) with three grinding beads (5/32" Craig Ball Sales) and ground for 1 min at 1100 strokes min⁻¹ in a genogrinder (SPEX SamplePrep). The procedure used by Greene et al. (2015) was used to confirm the presence of the GE gene in the seedlings.

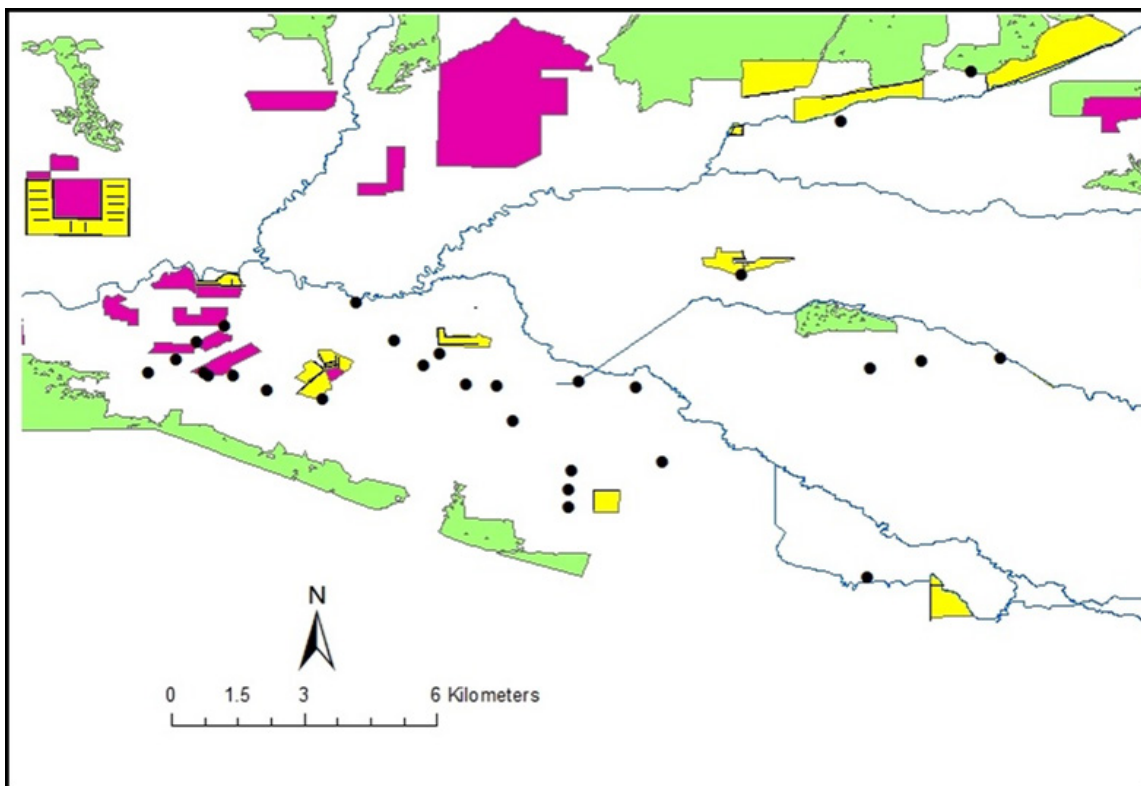


Fig. 1. Map shows the location of all commercial genetically engineered (GE) glyphosate-resistant seed fields but only shows conventional seed fields that we sampled for transgene presence in 2013 near Touchet Area, Walla Walla Valley, Washington. Pink represents GE glyphosate-resistant seed fields, yellow represents conventional seed fields, green represents rangeland, blue represents streams, black dots represent alkali bee beds, and dark black lines represent transects and edges sampled.

Data Analysis

Statistical Analysis

Whereas exponential decay models have been used to study the movement of transgenes into non-GE crops (Fitzpatrick et al., 2003; Teuber et al., 2004; Hagler et al., 2011; Scorza et al., 2013; Dong et al., 2016), we elected to use a quadratic regression approach, which has fewer assumptions and can assume a wider variety of decay shapes than an exponential model. We used a logit transformation on proportion of AP, $\log[p/(1 - p)]$, where a small amount of positive noise was added to zero counts to avoid taking the log of zero. The original data did not differ significantly from data simulated from the model, based on posterior predictive test results, with simulated data producing values similar to the original ones, based on centipede plots (Kramer 2014). We picked 0.1, 0.5, and 0.9% as threshold levels because the industry has established $<0.1\%$ as a nondetect standard and individual seed companies have adopted thresholds ranging from 0.1 to 0.9%. We used a 95% cutoff, since most of our samples were from the field edge; thus, estimated distances for the cutoff of the whole field would be conservative. Data analysis was performed using the R software (<http://www.r-project.org/>).

Spatial Mapping

Kriging was used to generate spatial prediction maps of AP proportion using the spatial statistics tools in ArcGIS 10.2 (ESRI, 2013). Kriging can be used to predict values at unmeasured locations, which are useful for creating contour maps and can be used to assess the uncertainty associated with a predicted value at an unmeasured location. Kriging was applied to

transformed AP proportions to create estimates over the entire study area on the logit scale using the Gaussian spatial model. Estimates were back-transformed to the proportion scale to create maps of spatial predictions and their standard errors.

RESULTS

The seedling germination assay described by Boyle et al. (2016) proved to be a cost-efficient way to measure low levels of AP in a large number of seed lots. In visual evaluation of seedlings, tolerant seedlings could be quickly identified based on root length and root hairs. These positive evaluations were consistently confirmed using test strips and PCR. The advantage of this assay is that it provides a quantitative measure and can be conducted at a relatively low cost, in terms of equipment and technical skill.

The relationship between AP proportion and distance to GE fields at 0.1, 0.5, and 0.9% AP threshold levels was modeled using the quadratic regression,

$$\text{logit}(p) = -3.775 - 0.0034[\log(\text{distance})] - 0.0798[\log(\text{distance})]^2$$

where $\text{logit}(p)$ is the logit of the AP proportion {i.e., $\log[p/(1 - p)]$ }, and $\log(\text{distance})$ is the log of distance from nearest GE field. The linear term $\log(\text{distance})$ was not significant ($p = 0.972$) but retained for parsimony; the $\log(\text{distance})^2$ coefficient was significant ($p < 0.0001$). Pollen-mediated gene flow rate was negatively ($r = -0.08$) correlated with

distance. The AP proportion decreased with increase in distance from GE fields. When threshold levels were set at 0.9% AP, the isolation distance needed between conventional and GE fields was 330 m to ensure at least 95% of the samples fell below this threshold level. When threshold levels were set at 0.5 and 0.1% AP, the required isolation distance between conventional and GE field was 602 and 2441 m to ensure that 95% of the samples fell below the 0.5 and 0.1% thresholds, respectively (Fig. 2). Since data from field edge samples were used in the analysis, we would expect that a random sample of seed taken throughout the field using these isolation distances would have AP at a lower level than the thresholds used here.

Spatial prediction maps were consistent with our statistical analysis results. Results indicate the AP was highest in the areas closest to GE seed production fields and lowest in the most distant fields (Fig. 3). At an average distance of 2.2 km, AP was 0.05%, and this level decreased to 0.03% at an average distance of 8.8 km from GE seed fields. The AP was higher near the riparian and open-range area and in fields located in the west and northwest sections of our study area, where GE seed fields tended to be clustered.

DISCUSSION

Our results documented transgene flow in commercial alfalfa seed production fields across a landscape that encompassed

270 km² and is the first study, to our knowledge, to examine transgene flow in an outcrossing crop that uses commercial pollinators on such a large geographic scale. Research at this scale has particular value considering the preponderance of evidence indicating that smaller scale research does not translate well to real-world situations (Amand et al., 2000; Rieger et al., 2002; Allnutt et al., 2008).

Similar to other studies (Fitzpatrick et al., 2003; Teuber et al., 2004; Fitzpatrick et al., 2007a; Hagler et al., 2011; Rieben et al., 2011; Scorza et al., 2013; Dong et al., 2016), results from our quadratic regression indicated that AP significantly decreased as the distance from GE fields increased. Results from our spatial prediction map showed similar results. However, our levels of AP tended to be higher than previous reports (Fitzpatrick et al., 2003; Teuber et al., 2007), which reinforced the importance of examining transgene flow on a landscape scale, as opposed to a research scale, to understand the risks of AP in conventional seed lots in an agroenvironment that supports both GE and non-GE seed production. Our predicted isolation distances suggested that the isolation distance recommended by NAFA for GE seed production using AB (1.6 km) should be increased to 2.5 km, to ensure neighboring conventional seed fields have 0.1% AP or less at a 95% confidence level. Our prediction map indicated gene flow of 0.03% at an average distance of

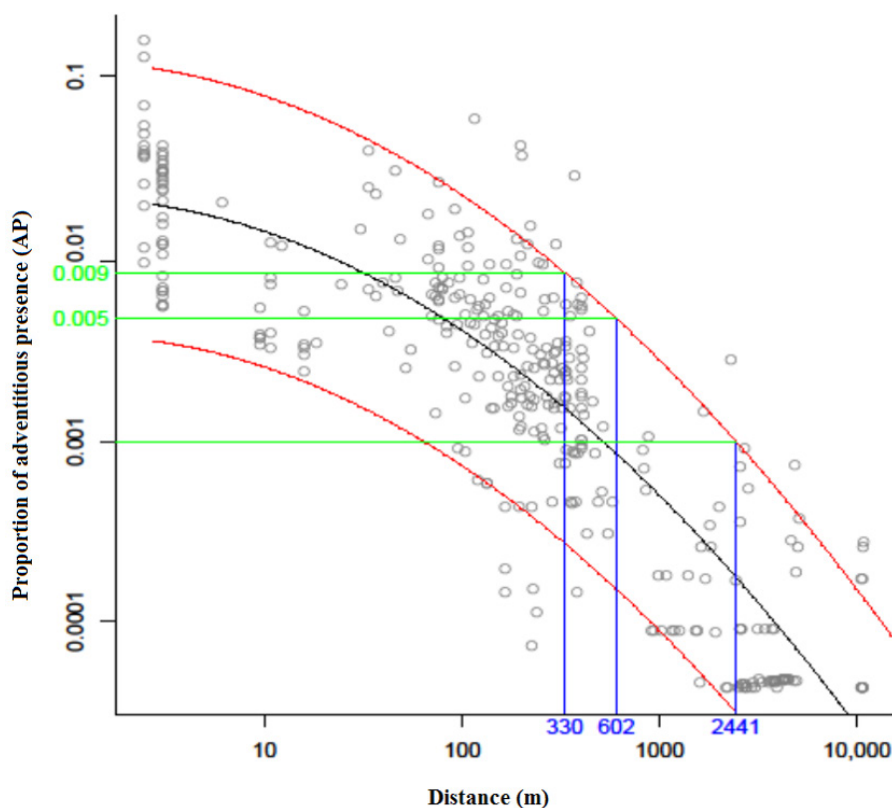


Fig. 2. Graph showing proportion of adventitious presence (AP) in relation to distance at different threshold levels (0.1, 0.5, and 0.9%). Black circles refer to observed proportion of AP at a given distance; the black line refers to model estimate (fitted line); the red line refers to 90% prediction interval on distance; the green horizontal lines represent the threshold levels of interested; the red and green line intersect is the distance (blue line).

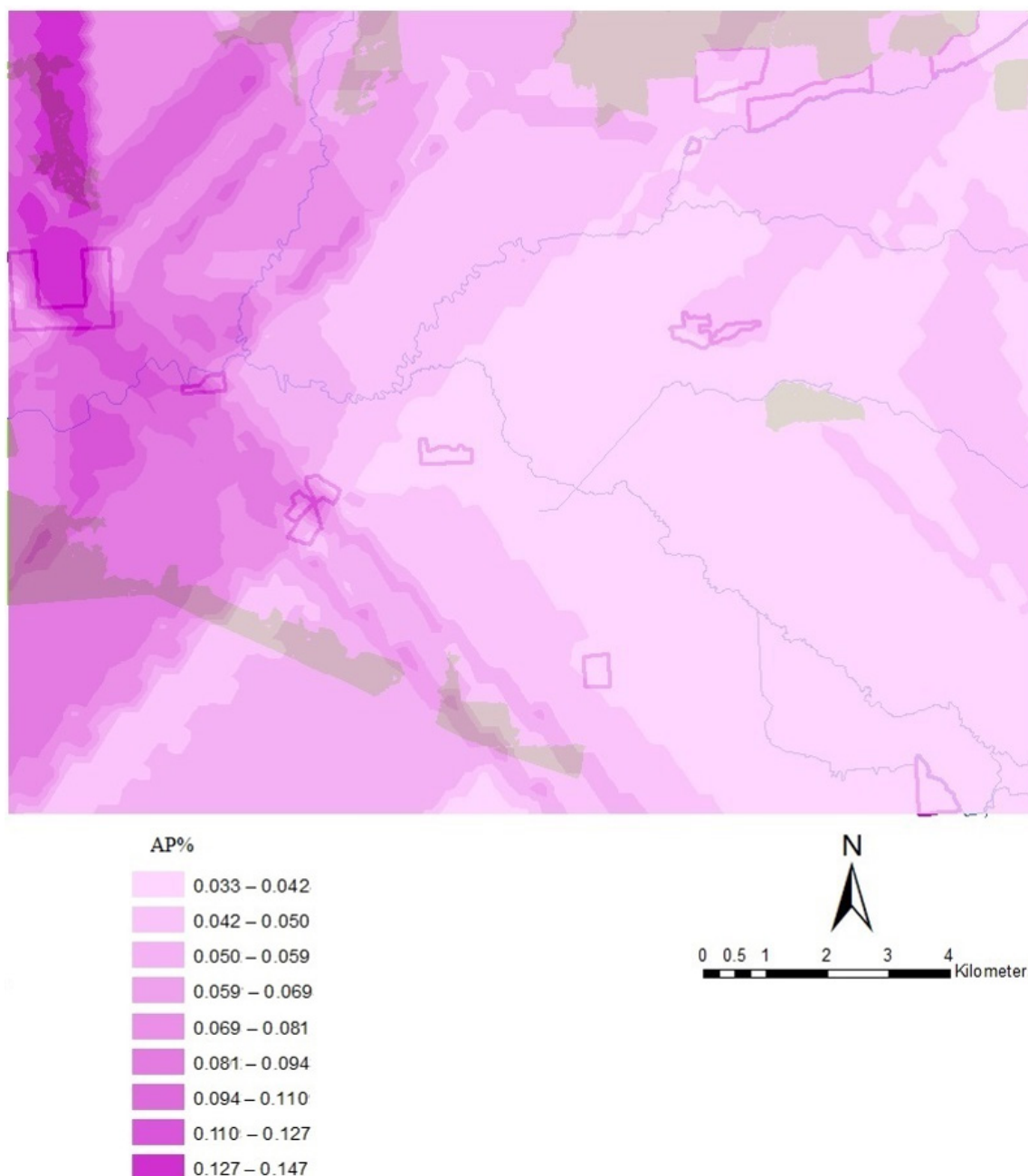


Fig. 3. Spatial prediction map with adventitious presence (AP) near Touchet Area, Walla Walla Valley, Washington. Light pink to dark pink refers to AP, dark pink outline refers to genetically engineered seed fields, dark green represents open-range area, and blue represents riparian area.

8.8 km from the closest GE seed fields, which supports the AOSCA ASSP standard of 8 km to ensure the production of alfalfa seed with very low levels of AP.

Establishing isolation distances to ensure coexistence of GE and non-GE alfalfa seed production is a challenging task, since gene flow is influenced not only by distance, but also by pollinators, not directly modeled here. Pollinator species differ in terms of physiology and behavior, which influences foraging distance, as well as interspecific competition, and response to climate, topography, and other environmental factors. However, physical distance is the single most important predictor of AP proportion

at the landscape scale (unpublished results). The results of this study emphasize the importance of using the appropriate scale to evaluate the impact of pollinator-mediated movement of transgenes. The results provide a set of isolation distances needed to ensure various AP thresholds, when a combination of ALCB and AB are used. Considering that AB have the greatest foraging distance of the commercial bee species used for alfalfa, the industry can apply these distance estimates as a conservative measure to manage the occurrence of AP. The isolation distances needed to ensure various AP thresholds, based on this landscape study, are greater than those recommended

based on smaller scale research trials. Although landscape-scale studies are more costly, they provide a better understanding of gene flow in the real world.

CONCLUSION

Successful coexistence between a crop that has GE and non-GE cultivars depends on having a clear understanding of how transgenes are likely to move in the environment and affect non-GE cultivars. For crops that are self-pollinated or otherwise limited in gene flow, coexistence is straightforward. However, for crops that are outcrossing and pollinated by insects, gene flow can be difficult to quantify. In turn, estimating suitable isolation distances can be problematic. This study demonstrates the importance of using a landscape-level scale to examine these issues. As further crops are developed that have GE and non-GE forms, adequate resources will ensure research can be conducted at the appropriate scale to develop coexistence strategies that support all growers.

Supplemental Material Available

The dataset used for analysis is available online for this article.

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